Weed Science, 52:91–97. 2004

Production of *Pleospora papaveracea* biomass in liquid culture and its infectivity on opium poppy (*Papaver somniferum*)

B. A. Bailey
Corresponding author. USDA-ARS, Alternate Crops and Systems Laboratory, Building 001, Room 342, Beltsville Agricultural Research Center-West, Beltsville, MD 20705; baileyb@ba.ars.usda.gov

K. P. Hebbar
R. D. Lumsden
USDA-ARS, Alternate Crops and Systems Laboratory, Building 001, Room 342, Beltsville Agricultural Research Center-West, Beltsville, MD 20705

N. R. O'Neill
USDA-ARS, Molecular Plant Pathology Laboratory, Building 004, Room 116, Beltsville Agricultural Research Center-West, Beltsville, MD 20705

J. A. Lewis
USDA-ARS, Alternate Crops and Systems Laboratory, Building 001, Room 342, Beltsville Agricultural Research Center-West, Beltsville, MD 20705

The fungus *Pleospora papaveracea* is a potential biocontrol agent for opium poppy. The objective of this study was to characterize the growth and production of propagules of *P. papaveracea* on various substrates and determine their infectivity on opium poppy. *Pleospora papaveracea* was grown on agar media containing wheat bran, corn cobs, soy fiber, cottonseed meal, rice flour, cornstarch, pectin, dextrin, or molasses, all with the addition of brewer's yeast (BY). Maximum radial growth of *P. papaveracea* occurred on molasses, soy fiber, and wheat bran media. *Pleospora papaveracea* produced chlamydospores on dextrin–BY and cornstarch–BY only. *Pleospora papaveracea* growth in liquid media with 1% (wt/v) dextrin, cornstarch, soy fiber, or wheat bran resulted in the production of greater than 10^6 colony-forming units (cfu) ml^{-1} within 3 to 5 d of incubation. *Pleospora papaveracea* produced less than 10^5 chlamydospores ml^{-1} after 10 d of incubation in wheat bran–BY and soy fiber–BY liquid media compared with the production of greater than 10^5 chlamydospores ml^{-1} after 5 d of incubation in dextrin–BY or cornstarch–BY liquid media. Fewer cfu were produced by *P. papaveracea* in 0.25% dextrin or 0.25 and 0.50% soy fiber liquid media than with 1 or 2% substrate. Greater than 10^7 chlamydospores g^{-1} dry weight and 10^8 cfu g^{-1} dry weight of *P. papaveracea* were produced in dextrin–BY liquid media in a commercial bench-top fermentor. After air drying biomass for 6 d, propagules of *P. papaveracea* remained infective on opium poppy. Mycelia and chlamydospores of *P. papaveracea* grew and formed appressoria during the infection process. Air-dried biomass, when rehydrated in 0.001% Tween 20, caused necrosis within 48 h after application to detached opium poppy leaves. At least 94% of the propagules from air-dried biomass that germinated and infected detached opium poppy leaves were of mycelial origin.

Nomenclature: Opium poppy, *Papaver somniferum* L.

Key words: *Pleospora papaveracea*, bioherbicide, biological control, formulation technology.

The fungus *Pleospora papaveracea* is being evaluated for possible use as a bioherbicide for the control of illicit opium poppy production (Bailey et al. 2000). *Pleospora papaveracea* is an ascomycete that produces conidia and chlamydospores (Farr et al. 2000); it infects opium poppy parts above and below ground causing seedling damping-off, girdling infections of the roots, and spotting of the leaves, stems, and capsules (Meffert 1950; O'Neill et al. 2000). The availability of adequate fungal biomass abundant in propagules that incite disease upon delivery (Boyetchko 1997; Boyette et al. 1991; Churchill 1982) is required if the fungus is to be successfully used as a bioherbicide.

Liquid, deep-tank fermentation, using inexpensive, readily available nutrient substrates, has been the industrial foundation for production of fungal propagules for several decades (Bowers 1982; Churchill 1982; Van Brunt 1986). This method of fermentation has been used to produce propagules of bioherbicidal fungi including *Colletotrichum gloeosporioides* f. sp. *aeschynomene* for control of northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] (Templeton et al. 1984) and *Fusarium oxysporum* f. sp. *erythroxyli* for control of coca [*Erythroxylam coca* (Lam.)] (Hebbar et al. 1997). Fermentor-produced propagules can be incorporated in a solid matrix to formulate granules, prills, and pellets for direct delivery to soil (Boyette et al. 1991; Fravel et al. 1998) or can be applied as a liquid suspension to coat leaf and stem surfaces (Boyette et al. 1991). For example, conidia of *Colletotrichum gloesporioides* are used in the commercial preparation of Collego®, which is applied as a foliar spray over rice fields to control northern jointvetch (Templeton et al. 1984). Recently, suspensions of *Dendryphion penicillatum* and *P. papaveracea* conidia were tested in the greenhouse and the field for the ability to infect opium poppy and cause disease (Bailey et al. 2000; O'Neill et al. 2000).

Techniques for the production of conidia of *P. papaveracea* on solid media are cumbersome and limit their use in mycoherbicides (Bailey et al. 2000; O'Neill et al. 2000). The objective of this study was to characterize the growth and production of propagules of *P. papaveracea* on various substrates and determine their infectivity on opium poppy.

Materials and Methods

Fungal Culture

The isolate of *P. papaveracea*[1] used in this study was virulent and aggressive against the opium poppy in growth chamber and field trials (Bailey et al. 2000; O'Neill et al.

2000). In culture, *P. papaveracea* produces septate conidia (Farr et al. 2000; Meffert 1950). *Pleospora papaveracea* also produces melanized thick-walled structures that are considered to be chlamydospores (Farr et al. 2000). In culture, *P. papaveracea* was grown on potato dextrose agar.[2] *Pleospora papaveracea* was stored as a suspension of hyphae and spores in 50% glycerol in water (v/v) at −20 C.

Growth of P. papaveracea *on Agar Media*

The ability of *P. papaveracea* to grow and sporulate on agar media was evaluated on several commercially available substrates. The substrates are inexpensive and have the potential for use in industrial fermentation systems for large-scale biomass production. The substrates included molasses,[3] wheat bran,[4] pectin,[5] rice flour,[6] dextrin,[7] cornstarch,[8] soy flour,[9] corn cobs,[10] cottonseed meal,[11] and brewer's yeast (BY).[12] The materials were used as purchased or processed to pass a 425-μm-mesh screen. Tap water (500 ml) was added to 1-L Erlenmeyer flasks with 5.0 g of each substrate, except BY and agar, which were added to each flask at 1.5% wt/v. The flasks were capped and autoclaved for 45 min on two consecutive days. The substrate suspensions were aseptically pipetted into 9-cm-diam sterile petri dishes (15 ml per petri dish).

One 7-mm-diam plug of *P. papaveracea* growing on PDA was centrally placed on the agar in each dish. The petri dishes were maintained under light at 22 to 25 C in a culture room. Radial mycelial growth (cm^2) of *P. papaveracea* on each plate was measured daily. After 6 d of growth, the area of agar with the colony was removed from the plate and blended with a Euroturrax[13] T20b homogenizer in 50 ml of 1% Triton 100-X solution for 1 min. The pH of the solution was determined, dilutions were prepared, and the concentration of conidia and chlamydospores was determined microscopically with a hemacytometer and calculated as numbers per square centimeter using the 6-d radial growth measurement.

Growth of P. papaveracea *in Liquid Media in Flasks*

Dextrin, cornstarch, wheat bran, and soy fiber, each supplemented with BY, were chosen for further study as substrates in liquid media. Factors examined in liquid media included the effect of *P. papaveracea* on substrate pH, the production of colony-forming units (cfu) as a measure of viability, and the development of conidia and chlamydospores. The substrates (1.0% wt/v) were placed in 250-ml Erlenmeyer flasks with 100 ml tap water and supplemented with BY (0.5% wt/v). The flasks were capped with porous foam plugs and autoclaved as indicated above. The flasks were inoculated with 1-ml aliquots of a homogenized agar plate suspension of *P. papaveracea*. Flasks were incubated at 22 to 25 C in the dark on a rotary shaker (125 rpm) for 10 d. In related experiments, the effects of substrate concentrations on fungal growth were determined using soy fiber–BY and dextrin–BY at rates of 0.25, 0.5, 1.0, and 2.0% wt/v, with all other parameters remaining the same.

Flask contents were homogenized with an Euroturrax T20b for 1 min after 0, 3, 5, and 10 d of growth by *P. papaveracea*. Aliquots of blended samples were diluted serially, and the numbers of spores formed were determined with a hemacytometer. Aliquots of dilutions were placed on PDA containing streptomycin and chlorotetracycline each at 50 μg ml^{-1} to prevent bacterial contamination. Within 4 to 5 d, the characteristic tan-brown colonies of *P. papaveracea* developed. Serial dilutions giving between 5 and 50 cfu $plate^{-1}$ or 5 and 50 conidia or chlamydospores per hemacytometer grid were used to determine the numbers of cfu, conidia, and chlamydospores per milliliter of medium.

Growth of P. papaveracea *in Bench-top Fermentors*

Pleospora papaveracea was grown in a 2.5-L commercial bench-top fermentor.[14] Dextrin (30.0 g) and BY (15.0 g) were autoclaved in 3-L of water for 40 min on two consecutive days. When cool, 2.5 L of the medium was transferred to the fermentor and then autoclaved an additional 30 min. A 20-ml aliquot of a 3-d-old starter flask of *P. papaveracea* was added to the fermentor. The fermentor medium temperature was maintained at 25 C. The agitation speed was adjusted to 200 rpm, and the fermentation was continued for 7 d. Air was sparged into the fermentor at the rate of 2 L min^{-1} of filtered air. *Pleospora papaveracea* was cultured three times. Ten-milliliter samples were withdrawn periodically, blended, and assayed for cfu and chlamydospores as described above. In addition, the total numbers of fungal propagules (mycelia, conidia, and chlamydospores combined) were counted in the same manner as chlamydospores.

After 7 d of incubation, the biomass was collected by filtering through a cheesecloth, and the excess moisture was removed. A subsample was oven dried overnight at 70 C to determine dry weight. The remaining biomass was divided into 1-g wet weight samples and air dried in a fume hood for 0, 1, and 6 d. Three samples per drying time were soaked in 10 ml of 0.001% Tween 20 for 30 min, blended as described above, and assayed for cfu and production of total fungal propagules and chlamydospores. Ten-microliter aliquots of dilutions (10^{-1} to 10^{-5}) of the blended samples were spotted onto detached opium poppy leaves. The leaves were incubated on wet #2 Whatman paper in petri dishes placed in sealed plastic bags to maintain humidity and to allow infection to occur. After 48 h, the poppy leaves were sectioned, stained with lactophenol cotton blue, and observed under a microscope for fungal germination and appressoria formation. Each germinated fungal propagule was characterized as originating from a mycelial fragment or a chlamydospore. The data are presented as the percentage of germinated fungal propagules forming appressoria, the percentage of germinated fungal propagules from mycelial fragments, and the percentage of total appressoria produced by mycelial fragments. In addition, the percent necrosis caused by each dilution was assessed as a percentage of the treated area showing necrosis. Necrosis numbers greater than 100% indicated that necrosis had expanded beyond the treated area (for example, 200% represents an area twice the size of the original treated area).

Statistical Analyses

The data from shake flask studies and necrosis measurements from the bench-top fermentation studies were analyzed using the SAS general linear models procedure repeated-measures analysis of variance[15] with time and a second factor (i.e., growth medium, growth medium concentration, or biomass drying time). Where interactions with

TABLE 1. Radial growth, propagule production, and pH values of *Pleospora papaveracea* on selected media.

Medium[a]	Radial growth[b]	Conidia[b]	Chlamydospores[b]	pH[b]
	cm^2	$\log_{10}((\text{no. } cm^{-2}) + 1)$		
Molasses	26 a[c]	4.6 de	0 b	5.8 b
Pectin	6 d	5.6 bc	0 b	4.0 d
Cornstarch	11 c	4.0 e	4.0 a	4.7 c
Dextrin	15 bc	4.0 e	4.0 a	4.1 cd
Rice flour	11 c	4.8 d	0 b	4.0 d
Soy fiber	28 a	6.3 a	0 b	7.8 a
Corn cob	16 b	5.0 cd	0 b	5.4 b
Wheat bran	25 a	5.8 ab	0 b	7.7 a
Cottonseed meal	19 b	4.8 d	0 b	5.7 b

[a] *Pleospora papaveracea* was grown for 6 d in agar culture on each substrate (1%) plus brewer's yeast (1.5%).

[b] The colony radius was measured and used to calculate the colony area (cm^2), and then the area of agar with the colony was removed from the plate and blended with a Euroturrax T20b[7] homogenizer in 50 ml of a 1% Triton 100-X solution. The pH of the solution was determined, and the amounts of conidia and chlamydospores were determined microscopically with a hemacytometer.

[c] Means within columns followed by the same letter are not significantly different ($P = 0.05$).

time were significant, means separation was carried out based on analysis of variance at each time point. The remaining data were analyzed using analysis of variance with single factors. The means separation was accomplished using least significant differences ($LSD_{0.05}$). Unless otherwise indicated, data for cfu, total fungal propagules, and chlamydospores were transformed using $\log_{10}([\text{no. ml}^{-1}] + 1)$ before analysis. Commercial fermentor data involving dried samples were transformed using $\log_{10}([\text{no. g}^{-1} \text{ dry weight}] + 1)$ before analysis. All experiments included four replications, except where indicated, and were repeated at least twice.

Results and Discussion

Development of *P. papaveracea* on Agar Media

The greatest amount of radial growth by *P. papaveracea* occurred on molasses–BY, soy fiber–BY, and wheat bran–BY agar medium (Table 1). The greatest number of conidia of *P. papaveracea* (cm^{-2}) was produced on soy fiber–BY agar medium. Chlamydospores were only produced on cornstarch–BY and dextrin–BY agar media. The pH of the agar media after 6 d of fungal growth varied from 4.0 to 7.8 (Table 1). The pH values of media at the time of fungal inoculation varied considerably from 4.0 for pectin–BY to 6.5 for cottonseed meal–BY (data not shown). The pH of soy fiber–BY and wheat bran–BY increased more than two units in response to the growth of *P. papaveracea*. The growth of *P. papaveracea* on the other media decreased acidity less than 0.6 unit.

Growth of *P. papaveracea* in Liquid Media in Shake Flasks

Experiments were performed in liquid culture with *P. papaveracea* using the four substrates, dextrin, cornstarch, soy fiber, and wheat bran. The substrates dextrin and cornstarch were selected because they promoted chlamydospore production on agar plates (Table 1) and are inexpensive, chemically homogeneous agricultural products. Soy fiber and wheat bran promoted conidia production and mycelial growth on agar plates (Table 1) and represent widely available heterogeneous processed plant tissues. The substrates in liquid media formed suspensions rather than solutions.

The observed change in pH of the four liquid media resulting from growth of *P. papaveracea* during 10 d of incubation was similar to that observed from growth of *P. papaveracea* on the agar media with these substrates. At the time of inoculation, the pH values of dextrin–BY, cornstarch–BY, soy fiber–BY, and wheat bran–BY were 4.1, 4.8, 4.5, and 5.4, respectively. During the 10-d growth period, soy fiber–BY and wheat bran–BY media became more alkaline (pH 7.5 to 8.2). However, the pH change in dextrin–BY and cornstarch–BY media was minimal and remained less than 5.5.

Pleospora papaveracea grew well on the four liquid media tested and developed 1×10^6 cfu ml^{-1} or more after 5 d of growth (Table 2). After incubating for 10 d, wheat bran–BY had greater cfu levels than soy fiber, which had greater cfu levels than cornstarch–BY and dextrin–BY (Table 2). Chlamydospores formed only in cornstarch–BY and dextrin–BY, reaching concentrations greater than 6×10^5 chlamydospores ml^{-1} culture medium (Table 2). Chlamydospore production was initiated between 3 and 5 d of incubation. Production of conidia by *P. papaveracea* in liquid media was only observed in soy fiber–BY and wheat bran–BY media and did not exceed 10^4 conidia ml^{-1} (data not shown).

Increasing concentrations of soy fiber resulted in faster

TABLE 2. Growth of *Pleospora papaveracea* on wheat bran–BY, cornstarch–BY, soy fiber–BY, and dextrin–BY in liquid media.[a]

	cfu				Chlamydospores			
Medium[b]	0 d	3 d	5 d	10 d	0 d	3 d	5 d	10 d
	$\log_{10}((\text{cfu ml}^{-1} \text{ media}) + 1)$[c]				$\log_{10}((\text{chlamydospores ml}^{-1} \text{ media}) + 1)$[c]			
Wheat bran	3.65	5.88 a	6.17 a	6.92 a	0	0 a	0 c	0 b
Cornstarch	—	5.62 a	6.03 a	6.25 c	—	0 a	4.54 b	5.79 a
Soy fiber	—	5.61 a	6.16 a	6.62 b	—	0 a	0 c	0 b
Dextrin	5.53	6.04 a	6.12 a	c	0	5.52 a	5.86 a	a

[a] Abbreviations: BY, brewer's yeast; cfu, colony-forming units.

[b] *Pleospora papaveracea* was grown in 100 ml liquid shake culture of each medium (1%) plus BY (0.5%). Flask contents were homogenized, and aliquots were placed on potato dextrose agar plus antibiotics to determine cfu. Chlamydospore numbers were determined with a hemacytometer.

[c] The data collected for cfu and chlamydospores after 3, 5, and 10 d were converted to $\log_{10}((\text{no. ml}^{-1}) + 1)$ and analyzed using repeated-measures analysis of variance. The culture age and medium interaction was significant for both cfu ($P \leq 0.048$) and chlamydospores ($P \leq 0.0001$). Means for cfu and chlamydospores within columns followed by the same letter are not different ($LSD_{0.05}$).

TABLE 3. Influence of substrate concentration on cfu and chlamydospore production of *Pleospora papaveracea* grown in soy fiber–BY and dextrin–BY liquid media.[a]

Medium[b]	Concentration	cfu[c]				Chlamydospores[c]			
		0 d	3 d	5 d	10 d	0 d	3 d	5 d	10 d
	% wt v^{-1}	$\log_{10}$ ((cfu ml^{-1} media) +1)				$\log_{10}$ ((chlamydospores ml^{-1} media) +1)			
Soy fiber	0.25	3.59	4.87 c	5.12 c	5.59 c	0	0	0	4.42 a
	0.50	—	5.34 b	5.73 b	5.85 b	—	0	0	4.20 a
	1.00	—	5.51 b	5.73 b	6.45 a	—	0	0	0 b
	2.00	—	6.10 a	6.52 a	6.33 a	—	0	0	1.33 b
Dextrin	0.25	4.21	4.37 c	5.42 b	5.33 b	0	0	4.76 a	5.00 b
	0.50	—	4.81 ab	6.04 a	6.25 a	—	0	5.00 a	5.70 a
	1.00	—	5.12 a	6.01 a	6.08 a	—	0	4.37 a	5.44 a
	2.00	—	4.72 b	6.12 a	6.34 a	—	0	3.66 b	5.63 a

[a] Abbreviations: BY, brewer's yeast; cfu, colony-forming units.
[b] *Pleospora papaveracea* was grown in 100 ml liquid shake culture of each medium at four concentrations plus brewer's yeast (0.5%). Flask contents were homogenized, and aliquots were placed on potato dextrose agar plus antibiotics to determine cfu. Chlamydospore numbers were determined with a hemacytometer.
[c] The data collected for cfu and chlamydospores after 3, 5, and 10 days was converted to $\log_{10}$((no. ml^{-1}) + 1) and analyzed using repeated-measures analysis of variance. The culture age and concentration interaction was significant for both cfu (soy fiber, $P \leq 0.0001$; dextrin, $P \leq 0.0048$) and chlamydospores (soy fiber, $P \leq 0.0001$; dextrin, $P \leq 0.0001$) in both media. Within median, means for cfu and chlamydospores within columns followed by the same letter are not different ($LSD_{0.05}$).

growth rates for *P. papaveracea* (Table 3), resulting in greater values for cfu (Table 3). Chlamydospore production was limited in soy fiber, with chlamydospores being observed only after 10 d of incubation (Table 3). The chlamydospore production was less for 1.0 and 2.0% soy fiber compared with chlamydospore production in 0.25 and 0.50% soy fiber (Table 3). *Pleospora papaveracea* grew at similar rates in 0.5, 1.0, and 2.0% dextrin (Table 3). There was a delay in chlamydospore production with 2% dextrin compared with 0.25, 0.50, and 1% dextrin (Table 3). After 10 d of incubation, *P. papaveracea* in 0.5, 1.0, and 2.0% dextrin plus BY produced similar chlamydospore concentrations.

Growth of *P. papaveracea* in Bench-top Fermentation

In bench-top fermentors, the cfu concentration of *P. papaveracea* increased from 128 cfu ml^{-1} media or less to 1×10^6 cfu ml^{-1} media or more after only 4 d of incubation, reaching a plateau between 4 and 6 d of incubation (Figure 1). With the bench-top fermentor, biomass of the *P. papaveracea* began accumulating on the walls of the vessel by 4 d, likely resulting in an underestimation of cfu production after 4 d. Production of mature chlamydospores by *P. papaveracea* began between 3 and 4 d of incubation and likewise plateaued between 4 and 6 d of incubation above 1×10^6 chlamydospores ml^{-1} media (Figure 1). The observed number of fungal propagules after grinding was approximately 1 $\log_{10}$ value larger than the measured cfu (Figure 1) at all time points. Similarly, before and after drying the biomass, the measured cfu was approximately 1 $\log_{10}$ less than the total fungal propagules observed (Figure 2). No more than one in 10 fungal propagules included a chlamydospore (Figure 2). The cfu per gram dry weight dropped 0.5 $\log_{10}$ units with drying, most of which occurred between 1 and 6 d (Figure 2). The biomass produced in the bench-top fermentor contained propagules that were infective on detached leaves of opium poppy under artificial humidity conditions provided in the laboratory, causing necrosis within 48 h of treatment (Figure 3). The propagules within *P. papaveracea* biomass diluted to 85 μg dry weight ml^{-1} caused approximately 75% necrosis (Figure 3) whether air dried for 0 (91.4% moisture), 1 (13.8% moisture), or 6 d (9.6% moisture). More than 125% necrosis was observed when biomass was applied in concentrations of 850 μg dry weight ml^{-1}, whereas little or no necrosis was observed when biomass was applied in concentrations of 8.5 μg dry weight ml^{-1}. More than 94% of the fungal propagules that germinated were of mycelial origin for both wet and dry biomass (Figure 4). From 45 to 60% of the germinated fungal propagules formed typical appressoria, with more than 94% of the appressoria being formed from mycelial fragments. Chlamydospores germinated and formed appressoria, but

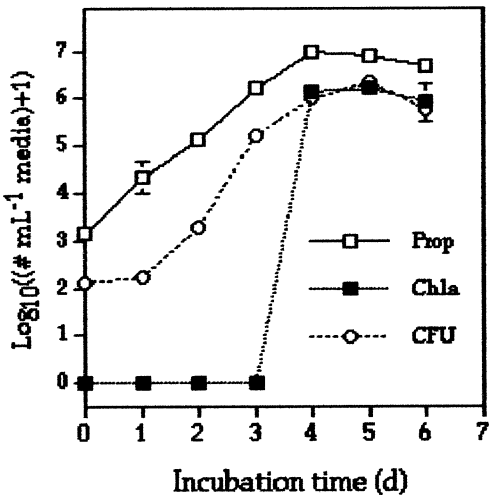

FIGURE 1. Production of fungal propagules, chlamydospores, and colony-forming units (cfu) by *Pleospora papaveracea* in a 2.5-L commercial bench-top fermentor. Ten-milliliter samples were withdrawn daily for 6 d, blended, and assayed for cfu and chlamydospore (Chla) production. Total fungal propagules (Prop), which includes both mycelial fragments and chlamydospores, also were counted. The data are presented at $\log_{10}$([(no. ml^{-1} media] + 1).

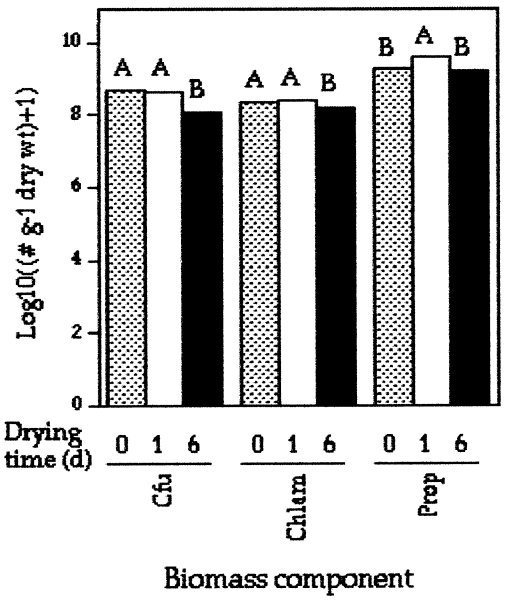

FIGURE 2. Effect of drying time on production of colony-forming units (cfu), chlamydospores, and fungal propagules produced by *Pleospora papaveracea* in a 2.5-L commercial bench-top fermentor. The biomass was collected after 7 d of growth and air dried for 0, 1, or 6 d. The dried biomass was blended and assayed for cfu and chlamydospore (Chlam) production. In addition, total fungal propagules (Prop), which includes both mycelial fragments and chlamydospores, were counted. Means within biomass component having the same letter are not different based on means separation using $LSD_{0.05}$.

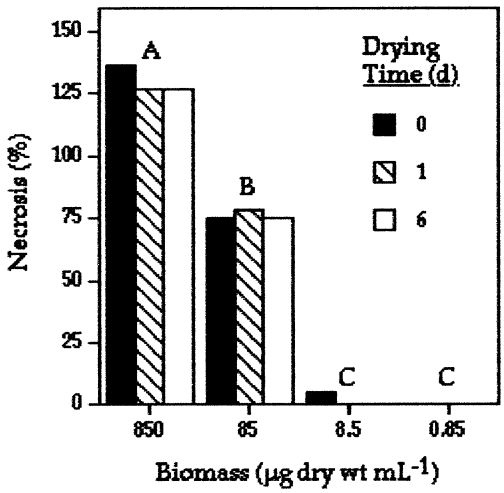

FIGURE 3. Effect of fungal biomass drying time on necrosis of opium poppy caused by *Pleospora papaveracea* biomass produced in a 2.5-L commercial bench-top fermentor. The fungal biomass was collected after 7 d of incubation and air dried for 0, 1, or 6 d. Ten-microliter aliquots of dilutions (10^{-1} to 10^{-5}) of the blended samples were spotted onto detached opium poppy leaves. After 48 h, the percent necrosis caused by each dilution was assessed as a percentage of the treated area showing necrosis. Although means for drying times are presented, the drying time (biomass) interaction was not significant ($LSD_{0.05}$). The mean necrosis for biomass (μg dry weight ml^{-1}) having the same letter is not different based on means separation using $LSD_{0.05}$.

they were a minority of infective propagules (< 5%), even after the biomass had air dried at room temperature for up to 6 d.

The successful implementation of biological control depends on the development of an effective formulation, which in turn, is dependent on the production of readily produced biomass of the biocontrol agent (Boyette et al. 1991; Churchill 1982; Greaves et al. 1998). It is also important that the substrate used for biomass production be inexpensive and widely available (Boyette et al. 1991; Churchill 1982; Hebbar et al. 1996; Papavizas et al. 1984). This report describes the development of infective propagules of *P. papaveracea* on these types of materials. All the materials that were extensively used (soy fiber, wheat bran, dextrin, and cornstarch) represent high-carbon substrates with different rates of decomposition. Soy fiber–BY, wheat bran–BY, dextrin–BY, and cornstarch–BY yielded high cfu for *P. papaveracea*. Chlamydospores of *P. papaveracea* were readily produced in dextrin–BY and cornstarch–BY and rarely produced in soy fiber–BY and wheat bran–BY. The bioherbicidal fungus *F. oxysporum* f. sp. *erythroxyli* produced low chlamydospore counts when grown on substrates with high-utilizable carbon (such as dextrin and cornstarch) and high chlamydospore and conidia numbers when grown on substrates with low-utilizable carbon (such as soy fiber and wheat bran) (Hebbar et al. 1996, 1997). Substrates with high carbon to nitrogen ratio supported chlamydospore formation by *F. oxysporum* f. sp. *elaeidis* (Oritsejofor 1986). The substrates used in this study had high carbon to nitrogen ratios but had variable effects on chlamydospore production.

Application of bioherbicides as foliar sprays may be most appropriate for biocontrol of an annual herbaceous plant

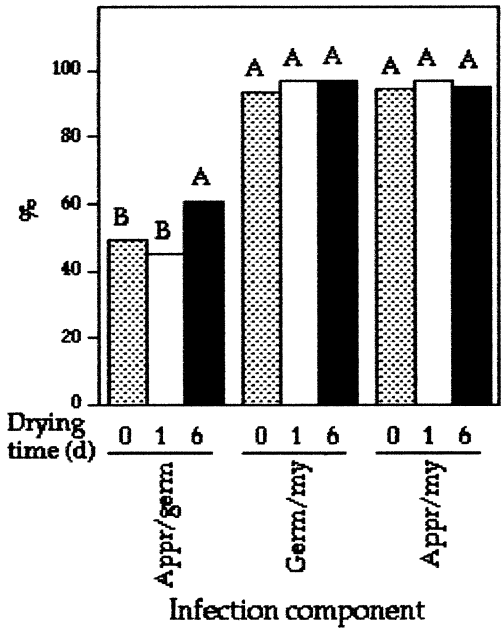

FIGURE 4. Effect of drying time on viability of fungal propagules and chlamydospores of *Pleospora papaveracea* produced in 2.5-L commercial bench-top fermentors. The biomass was collected after 7 d and air dried for 0, 1, or 6 d. Ten-microliter aliquots of dilutions (10^{-1} to 10^{-5}) of the blended samples were spotted onto detached opium poppy leaves. After 48 h, each germinated fungal propagule was characterized as originating from mycelia or chlamydospores. The data are presented as the percentage of germinated fungal propagules forming appressoria (Appr/germ), the percentage of germinated fungal propagules of mycelial origin (Germ/my), and the percentage of total appressoria formed that were of mycelial origin (Appr/my). Means within propagule components having the same letter are not different based on means separation using $LSD_{0.05}$.

such as opium poppy (Boyette et al. 1991). With surfactants, the biomass of *P. papaveracea* produced by liquid fermentation could be applied in a manner similar to conidial formulations (Bailey et al. 2000). Although conidia were produced in liquid shake flask cultures of *P. papaveracea*, their numbers were low, and they originated from mycelia attached to the flask above the liquid media. The production of conidia in liquid culture can be problematic for many fungi (Boyette et al. 1991) as is the case for *P. papaveracea*. Our demonstration that both mycelial propagules and chlamydospores infect opium poppy in a manner similar to that reported for conidia (Bailey et al. 2000) expands the potential types of propagules that may be considered for inclusion in foliar-applied formulations.

The fermentation time required for the production of biomass is important for the development of biocontrol agents. This was addressed by Lumsden et al. (1996) with regard to the minimal amount of time needed to produce chlamydospores of *Trichoderma virens*. Large-scale production of chlamydospores of *F. oxysporum* isolates could require 5 wk for solid-state fermentation (Hildebrand and McCain 1978) or as little as 2 wk for liquid fermentation (Hebbar et al. 1997). Production of *P. papaveracea* chlamydospores and cfu in dextrin and cornstarch media continued to increase up to 10 d of fermentation (Tables 2 and 3). In addition to the use of culture flasks, appreciable cfu and chlamydospore formation was achieved in commercial bench-top fermentors (Figure 1). Although liquid fermentations in previous studies with isolates of *Trichoderma* spp. were allowed to proceed 9 d before harvest of biomass, chlamydospores of *P. papaveracea* formed readily, requiring only 4 to 6 d of incubation (Papavizas et al. 1984).

Substrate quantities used in fermentation systems can be important with regard to cost of formulations with adequate quantities of effective propagules. In some cases, as was observed in this study for chlamydospores of *P. papaveracea*, a high substrate level can reduce or delay production of the required numbers of propagules. This also was observed with various substrates for *F. oxysporum* f. sp. *erythroxyli* (Hebbar et al. 1996) and *Alternaria alternata* (Osman et al. 1992).

The most commonly used propagules in bioherbicidal formulations have been spores–conidia largely because of their ease of production and handling (Boyetchko 1997; Boyette et al. 1991; Churchill 1982). The use of chlamydospores in bioherbicide formulations has been described in several systems (Hebbar et al. 1996, 1997), but the use of mycelial fragments as primary inoculum in a bioherbicide has been less studied. The biomass of *P. papaveracea* produced, using bench-top fermentors, consisted of both melanized and nonmelanized mycelial fragments and chlamydospores. A portion of the mycelial component of the biomass survived air drying for at least 6 d. The mycelial component of the biomass was the primary source of appressoria formation on detached opium poppy leaves. Chlamydospores, although capable of germinating and infecting opium poppy, were not a major source of infection with the biomass used in bioassays. Mycelia are generally considered more difficult to handle and store and in some cases are less aggressive than other inoculum sources (Boyette et al. 1991; Churchill 1982). Over the past several years, formulation and storage strategies have been developed for preservation of mycelia for bioherbicidal applications (Amsellem et al. 1999). Mycelia of *F. arthrosporioides* and *F. oxysporum*, pathogens of *Orobanche* spp., had good storage characteristics when incorporated into "Stabileze" formulations (Amsellem et al. 1999). Mycelia of *P. papaveracea* are the major portion of the biomass produced in liquid culture and will infect opium poppy plants. If mycelia are to be considered as a source of biomass for incorporation into a bioherbicide for opium poppy, methods for maintaining the viability of the mycelia need to be developed.

The results of this investigation demonstrated the development of the bioherbicidal fungus *P. papaveracea* from the agar plate stage to fermentation in a commercial apparatus. The data suggest the feasibility of growing biomass containing infective propagules of *P. papaveracea* capable of debilitating or destroying opium poppy (Bailey et al. 2000; O'Neill et al. 2000). Unique formulation and application technologies continue to be developed (Amsellem et al. 1999), which should enhance the opportunity to use mycelial–chlamydospore biomass in bioherbicides. Considerable research remains to be done with *P. papaveracea* regarding large-scale fermentation, delivery, shelf life, and potential formulations. However, this research shows that the biomass of *P. papaveracea* that is infective on opium poppy can be grown on inexpensive, readily available agricultural by-products.

Sources of Materials

[1] *Pleospora papaveracea*, Soybean and Alfalfa Research Laboratory, Beltsville Agricultural Research Center-West, 10300 Baltimore Boulevard, Beltsville, MD 20705.

[2] Potato dextrose agar, Difco Laboratories, P.O. Box 331058, Detroit, MI 48232.

[3] Molasses, USDA Grainery, 10300 Baltimore Boulevard, Building 177C, Beltsville Agricultural Research Center-East, Beltsville, MD 20705.

[4] Wheat bran, USDA Grainery, 10300 Baltimore Boulevard, Building 177C, Beltsville Agricultural Research Center-East, Beltsville, MD 20705.

[5] Pectin, Holton Industries, Co., 1 Eighth Street, Frenchtown, NJ 08825.

[6] Rice flour, Holton Industries, Co., 1 Eighth Street, Frenchtown, NJ 08825.

[7] Dextrin, Holton Industries, Co., 1 Eighth Street, Frenchtown, NJ 08825.

[8] Cornstarch, CPC International, Inc., 6500 Archer Road, Argo, IL 60501.

[9] Soy flour, Lauhoff Grain Co., P.O. Box 571, Danville, IL 61834.

[10] Corn cobs, The Andersons, 530 Illinois Avenue, Maumee, OH 43537.

[11] Cottonseed meal, Pharmamedia®, Trader's Protein, P.O. Box 80367, Memphis, TN 38108.

[12] Brewer's yeast, Universal Foods Corporation, P.O. Box 737, Milwaukee, WI 53201.

[13] Euroturrax® T20b homogenizer, New Brunswick Scientific, 44 Talmadge Road, P.O. Box 4005, Edison, NJ 08818.

[14] Commercial bench-top fermentor (2.5 L), Bioflo IIC®, New Brunswick Scientific, 44 Talmadge Road, P.O. Box 4005, Edison, NJ 08818.

[15] SAS version 6.04, SAS Institute Inc., Campus Drive, Cary, NC 27513.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. De-

partment of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

Acknowledgments

We thank Sherry Slater and Stephen Poch for their excellent technical support.

Literature Cited

Amsellem, Z., N. K. Zidack, P. C. Quimby, Jr., and J. Gressel. 1999. Long term dry preservation of active mycelia of two mycoherbicidal organisms. Crop Prot. 18:643–649.

Bailey, B. A., P. C. Apel-Birkhold, N. R. O'Neill, J. Plaskowitz, S. Alavi, J. C. Jennings, and J. A. Anderson. 2000. Evaluation of infection processes and resulting disease caused by *Dendryphion penicillatum* and *Pleospora papaveracea* on *Papaver somniferum*. Phytopathology 90:699–709.

Bowers, R. C. 1982. Commercialization of microbial biological control agents. Pages 157–173 *in* R. Charudatan and H. L. Walker, eds. Biological Control of Weeds with Plant Pathogens. New York: J. Wiley.

Boyetchko, S. M. 1997. Principles of biological weed control with microorganisms. Hortscience 32:201–205.

Boyette, C. D., P. C. Quimby, Jr., W. J. Connick, Jr., D. J. Daigle, and F. E. Fulgham. 1991. Progress in the production, formulation, and application of mycoherbicides. Pages 209–222 *in* D. O. TeBeest, ed. Microbial Control of Weeds. New York: Chapman and Hall.

Churchill, B. W. 1982. Mass production of microorganisms for biological control. Pages 139–156 *in* R. Charudattan and H. L. Walker, eds. Biological Control of Weeds with Plant Pathogens. New York: J. Wiley.

Farr, D. F., N. R. O'Neill, and P. B. van Berkum. 2000. Morphological and molecular studies on *Dendryphion penicillatum* and *Pleospora papaveracea*, pathogens of *Papaver somniferum*. Mycologia 92:145–153.

Fravel, D. R., W. J. Connick, Jr., and J. A. Lewis. 1998. Formulations of microorganisms to control plant diseases. Pages 187–202 *in* H. D. Burges, ed. Formulation of Microbial Biopesticides. Dordrecht, The Netherlands: Kluwer Academic.

Greaves, M. P., P. J. Holloway, and B. A. Auld. 1998. Formulations of microbial herbicides. Pages 203–233 *in* H. D. Burges, ed. Formulation of Microbial Biopesticides. Dordrecht, The Netherlands: Kluwer Academic.

Hebbar, K. P., J. A. Lewis, S. M. Poch, and R. D. Lumsden. 1996. Agricultural by-products as substrates for growth, conidiation and chlamydospore formation by a potential mycoherbicide, *Fusarium oxysporum* strain EN-4. Biocontrol Sci. Technol. 6:263–175.

Hebbar, P. K., R. D. Lumsden, S. M. Poch, and J. A. Lewis. 1997. Liquid fermentation to produce biomass of mycoherbicidal strains of *Fusarium oxysporum*. Appl. Microbiol. Biotechnol. 48:714–719.

Hildebrand, D. C. and A. H. McCain. 1978. The use of various substances for large-scale production of *Fusarium oxysporum* f. sp. *cannabis* inoculum. Phytopathology 68:1099–1101.

Lumsden, R. D., J. F. Walter, and C. P. Baker. 1996. Development of *Gliocladium virens* for damping-off disease control. Can. J. Plant Pathol. 19:463–468.

Meffert, M. E. 1950. Ein Beirtag zur Biologie und Morphologie der Erreger der parasitaren Blattdurra des Mohns. Z. Parasitenkd. Bd. 14:442–498.

O'Neill, N. R., J. C. Jennings, B. A. Bailey, and D. F. Farr. 2000. *Dendryphion penicillatum* and *Pleospora papaveracea*, destructive seedborne pathogen and potential mycoherbicides for *Papaver somniferum*. Phytopathology 90:691–698.

Oritsejofor, J. J. 1986. Carbon and nitrogen nutrition in relation to growth and sporulation of *Fusarium oxysporum* f. sp. *elacides*. Trans. Br. Mycol. Soc. 87:519–524.

Osman, M., M. A. El-Sayed, Y.A.H. Mohamed, and M. Metwally. 1992. Effect of various culture conditions on *Alternaria alternata* and *Fusarium oxysporum*, 1. Culture media, temperature, age and carbon source. Microbios 71:15–26.

Papavizas, G. C., M. T. Dunn, J. A. Lewis, and J. Beagle-Ristaino. 1984. Liquid fermentation technology for experimental production of biocontrol fungi. Phytopathology 74:1171–1175.

Templeton, G. E., D. O. TeBeest, and R. J. Smith, Jr. 1984. Biological weed control in rice with a strain of *Colletotrichum gleosporiodes* (Penz.) Sacc. used as a mycoherbicide. Crop Prot. 3:409–422.

Van Brunt, J. 1986. Fermentation economics. Biotechnology 4:395–401.

Received January 28, 2003, and approved April 25, 2003.